Supplementary data: Characterization of human primary pre-osteoblasts

Prior experiments, pre-osteoblast primary cultures were characterized to confirm their phenotype. Morphological analysis, carried out on stained cells (Figure S, panel A), confirmed the ovoidal/polygonal shape and the dimension range (around 20-30µm in diameter) typical of osteoblasts. Population doubling time was calculated during subsequent culture passages and, even though variable among different primary populations, indicated a slow growth rate with a mean value of 177.8 ± 83.5 hours (*n*=13). The specific expression of bone ECM components, in particular Type I Collagen, Osteopontin, SPARC and Osteocalcin was validated by Western Blot analysis and/or immunofluorescence staining (panels B-D). The analysis of OB gene expression profile was performed in parallel with mesenchymal stem/stromal cells harvested from bone marrow (BMSCs) and adipose tissue (ASCs). OBs share with BMSCs similar expression levels of RUNX2 and KLF4, osteogenic and stemness markers, respectively. In addition, both cell populations are more osteo-commmitted and mature than ASCs (panel E). Finally, OB basal or osteo-induced mineralization potential was compared to BMSCs isolated from the same patients. We confirmed a pronounced osteogenic ability of OBs respect to their progenitor cells independently of the presence of osteoinductive factors (panel F).

Materials and methods:

Cell morphology was evaluated through DiffQuik staining (Medion Diagnostics, Miami, FL, USA), following the kit protocol.

Population doubling time (DT) was assessed as follows:

$$DT = \delta t \times \ln(2) / \ln(C/P)$$

where C and P represent the number of counted and plated cells respectively and δt the time span (hours) between subsequent culture passages.

Western Blot analysis was carried out following the procedure described in section *Analysis of matrix production* of the main text. In this set up, GAPDH (sc-20357, Santa Cruz Biotechnology, CA, USA, dilution 1:1000) was used as internal standard.

The expression of ECM proteins was further confirmed through immunofluorescence staining. Briefly, osteoblasts were seeded on round coverslips and cultured for a week. Samples were then rinsed twice with PBS, fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.1%Triton X-100 for 2 minutes. Primary antibodies raised against Osteocalcin (sc-30044, Santa Cruz Biotechnology, CA, USA, dilution 1:50) and Osteopontin (ab8448, Abcam, Hongkong, China, dilution 1:50) were incubated overnight at 4°C. After two washes with PBS additioned with 1%BSA, appropriate secondary antibodies conjugated to AlexaFluor 568 or 488 (Thermo Fisher Scientific, Waltham, MA, USA, dilution 1:400) were incubated at room temperature for 45 minutes. Cover slips were then mounted with ProLong® Diamond Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed through the Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan).

Gene expression of the osteogenic marker RUNX2 (Runt-related transcription factor 2) and the stemness marker KLF4 (Kruppel-like factor 4) was assessed by real-time polymerase chain reaction (RT-PCR, StepOne Plus, Life Technologies, Carlsbad, CA, USA). Briefly, total RNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was obtained through high-capacity cDNA reverse transcription kit. Amplicons were generated with Single Tube TaqMan® Gene Expression Assays using the probe hs00231692_m1 for RUNX2 and hs00358836_m1 for KLF4 (Applied Biosystems, Foster City, CA, USA). For each sample, data were normalized on β-Actin expression (ACTB, hs01060665_g1) and the relative quantification was determined using the delta delta CT (ΔΔCT) method in comparison to MG63 osteoblast-like cells (here used as landmark for cell commitment towards the osteogenic lineage).

Mineralization potential was assessed, following the procedure described in the main text, in comparison to coupled bone marrow mesenchymal stem/stromal cells isolated from the same patients, both in standard and osteogenic conditions (culture medium added with 100nM dexamethasone, 50µg/ml ascorbic acid and10mM β glycerophosphate, all reagents purchased from Sigma-Aldrich, St. Louis, MO, USA).

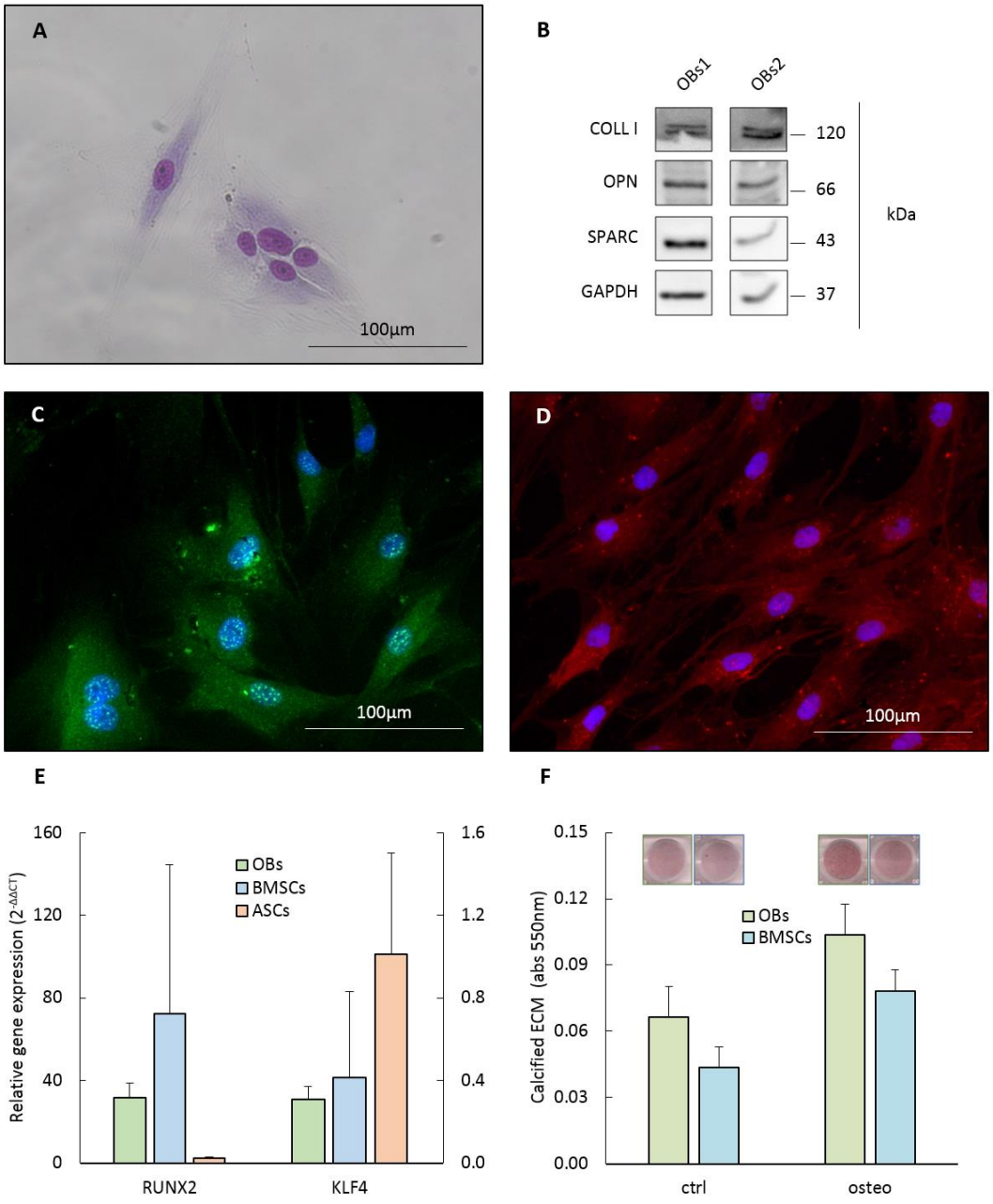

Figure S. Characterization of human primary pre-osteoblasts. (**A**) Morphology of fixed cells. (**B**) Expression of Type I Collagen, Osteopontin, SPARC and GAPDH by WB analysis. (**C**) and (**D**) Osteopontin (green) and Osteocalcin (red) expression in pre-osteoblasts by immunofluorescence. Nuclei were counterstained with DAPI. (**E**) Relative gene expression of RUNX2 and KLF4 in respect to MG63 cells by RT-PCR. mRNA levels of RUNX2 and KLF4 are relative to β-Actin, used as internal control. Data are expressed as mean ± SEM of 3 independent experiments for each cell type. (**F**) Calcified extracellular matrix deposition by OBs and BMSCs cultured in either standard (ctrl) or osteoinductive (osteo) conditions by Alizarin Red-S staining. Data are expressed as mean ± SEM of 4 independent experiments. Representative images of cells stained with Alizarin Red-S dye are shown.